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(54) Title: ENZYMATIC ANTIMICROBIAL COMPOSITIONS

(57) Abstract

   An antimicrobial composition is provided, comprising a Vanadium haloperoxidase, a source of halide and hydrogen peroxide or a source of hydrogen peroxide. Preferably, the Vanadium haloperoxidase is a chloroperoxidase obtainable from Curvularia inaequalis.

ENZYMATIC ANTIMICROBIAL COMPOSITIONS

TECHNICAL FIELD

5   The present invention relates to the field of
enzymatic antimicrobial compositions and their use. More in
particular, it relates to enzymatic antimicrobial
compositions comprising a Vanadium haloperoxidase, a source
of hydrogen peroxide and a source of halide. The invention
10   also relates to the production of Vanadium haloperoxidase
by means of recombinant DNA technique, which may be used in
enzymatic antimicrobial compositions.

15   BACKGROUND AND PRIOR ART
            Various enzymatic antimicrobial compositions are
known in the art. For instance, WO-A-94/04217 discloses
stabilized dentifrice compositions which are capable of
producing antimicrobially effective concentrations of
20   hypothiocyanite ions (OSCN⁻). The compositions contain an
oxidoreductase to produce hydrogen peroxide and a
peroxidase enzyme capable of oxidising thiocyanate ions,
which are normally present in saliva, to antimicrobial
hypothiocyanite ions (OSCN⁻). Suitable peroxidases include
25   lactoperoxidase, myeloperoxidase salivary peroxidase and
chloroperoxidase.
            Enzymatic antimicrobial compositions comprising a
haloperoxidase are also disclosed in EP-A-500 387
(Exoxemis). It is described that haloperoxidases
30   selectively bind to and inhibit the growth of target
microbes in the presence of peroxide and halide. As
suitable haloperoxidases EP-A-500 387 mentions
myeloperoxidase (MPO), eosinophil oxidase (EPO),
lactoperoxidase (LPO) and chloroperoxidase (CPO). It was
35   reported that the ratio of halide to hydrogen peroxide is a
critical factor with regard to the stability and
functionality of the haloperoxidase. At very low ratios,
hydrogen peroxide can inhibit the haloperoxidase function,
whereas at very high ratios, the halide can block the

enzymatic reaction. The ratio may vary over a broad range, but is preferably kept above about 50.

Because of unwanted side reactions of the hydrogen peroxide, the actual hydrogen peroxide

5   concentration in the antimicrobial composition might be lower than expected. Therefore, the present inventors found it more desirable to have the possibility to employ a high starting concentration hydrogen peroxide in the antimicrobial composition, in combination with a

10 conventional amount of halide.

Furthermore, there is a need for enzymatic antimicrobial compositions having a spectrum of antimicrobial activity which is different from that of the known enzymatic antimicrobial compositions. Preferably, the

15 compositions should be capable of exhibiting antimicrobial activity against micro-organisms which are difficult to combat, e.g. Streptococcus faecalis. In other circumstances it may be desirable to combat also non-pathogenic microorganisms, because they may cause spoilage of food

20 products.

Thus, the purpose of the present invention is to provide an enzymatic antimicrobial composition which obviates one or more of the above disadvantages.

We have now surprisingly found that particularly

25 effective enzymatic antimicrobial compositions can be formulated when Vanadium haloperoxidases are used.

The prior art mentioned above includes a variety of haloperoxidases for use in antimicrobial compositions, but no particular attention has been paid so far to the

30 class of Vanadium haloperoxidases.

Vanadium haloperoxidases are different from other haloperoxidases in that the prosthetic group in these enzymes has structural features similar to vanadate (vanadium V), whereas the other haloperoxidases are heme-

35 peroxidases.

A further purpose of the invention was to clone the gene encoding a Vanadium haloperoxidase and to determine its sequence to allow its expression in other

micro-organisms which are more convenient to grow and also
to increase the amount of enzyme which can be produced
using recombinant DNA techniques.

5   Some of the Vanadium bromoperoxidases are found
in nature on the surface of seaweeds (Wever et al. 1991).
In the intact plant in seawater the Vanadium bromo-
peroxidase is accessible to added substrates and is able to
release HOBr upon addition of hydrogen peroxide. The role
of the enzyme has not been established, but it is likely
10   that formation of the highly oxidizing HOBr in seawater is
part of a defense system of the plant to prevent microbial
or fungal growth on its surface.

       After the discovery of the non-heme Vanadium
bromoperoxidases from the seaweed Ascophyllum nodosum, it
15   was shown that a large number of other seaweed species were
also contains these enzymes. In particular, the bromo-
peroxidase from A. nodosum has been extensively studied and
characterized (for reviews, see references 2-3). The
prosthetic group in these enzymes has structural features
20   similar to vanadate (Vanadium V). In the catalytic
mechanism that was derived for this enzyme, hydrogen
peroxide reacts with the enzyme to form a hydrogen
peroxide-enzyme complex after which bromide and a proton
reacted with the complex to form an enzyme-HOBr complex. It
25   was shown (De Boer et al., 1988) that this complex decayed
to yield enzyme and HOBr. These vanadium bromo-peroxidases
were also shown (De Boer et al., 1988)) to have a high
operational stability in aqueous and organic media. For
example, these enzymes were stable for three weeks under
30   turnover conditions and could be stored for more than a
month in organic solvents such as acetone, methanol,
ethanol (present up to 60% v/v) and 1-propanol, without
loss of activity. However, these enzymes have the
disadvantage that for potential applications bromide should
35   be present or added and further attempts to clone the genes
coding for these bromo peroxidases from seaweeds and to
determine their amino acid sequences have not been
successful.

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The known existing heme-containing
chloroperoxidase from Caldariomyces fumago is l ss suitable
for preparing enzymatic antimicrobial compositions because
of its inherent instability and its low pH optimum of 2.75
5   (J.R. Kanofsky, 1984), which seriously limits its
application. Similar arguments prevent the application of
the enzyme myeloperoxidase (MPO) from human white blood
cells, which is also capable of generating HOCl (A.R.J.
Bakkenist et al., 1980).

10   Reports (see references 8, 9) have already
appeared that dematiaceous hyphomyctes secrete
haloperoxidases with pronounced stability. In particular it
was shown that the terrestrial fungus Curvularia inaequalis
secretes a Vanadium chloroperoxidase (J.W.P.M. Van
15   Schijndel et al, 1993) which possesses a high affinity for
chloride and has a pH optimum for the chlorination reaction
around pH 5.5. As with the bromoperoxidase from seaweed,
the prosthetic group in the chloroperoxidase has structural
features similar to vanadate (vanadium V). In a subsequent
20   more detailed study (J.W.P.M. Van Schijndel et al., 1994),
it was shown that the enzyme kinetics of the oxidation of
chloride by hydrogen peroxide resemble that of the vanadium
bromoperoxidase from the seaweed A. nodosum. Further, three
different methods showed that the enzyme produces an
25   oxidized chlorine species (HOCl) as reaction product and
was by itself resistant towards this product. The enzyme
exhibits a high thermostability (Tm 90°C) and displays high
stability in organic solvent such as 40 % methanol, ethanol
and propanol.

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DEFINITION OF THE INVENTION
            In a first aspect, the present invention relates
to an enzymatic antimicrobial composition comprising a
35   Vanadium haloperoxidase, a source of halide and hydrogen
peroxide or a source of hydrogen peroxide.
            According to a second aspect, the present
invention relates to the production of a Vanadium

haloperoxidase by transforming a suitable host by means of
an expression vector comprising an origin of replication,
transcription and termination control sequences and at
least part of the DNA sequence coding for a Vanadium
5   haloperoxidase, cultivating the host under conditions which
allow the expression of the structural gene and isolating
the Vanadium haloperoxidase.

10. DETAILED DESCRIPTION OF THE INVENTION
     (a) The Vanadium haloperoxidase
             The enzymatic antimicrobial compositions
     according to the invention comprise, as a first
     constituent, a Vanadium haloperoxidase. The Vanadium
15   haloperoxidase may in principle be chosen from the various
     Vanadium haloperoxidases which have been disclosed in the
     art. For example, one can use the Vanadium (non-heme)
     haloperoxidase produced from Curvularia inaequalis, such as
     described in US-A-4 707 466. Alternatively, one may isolate
20   and purify the chloroperoxidase from Curvularia inaequalis
     (CBS 102.42) according to the method of J.W.P.M. Van
     Schijndel et al. (1993).
             Other sources of Vanadium haloperoxidases include
     Drechslera biseptata (CBS 371.72), Drechslera fugax (CBS
25   509.77), Drechslera nicotiae (CBS 655.74), Drechslera
     subpapendorfii (656.74), Embelisia hyacinthi (416.71),
     Embelisia didymospora (CBS 766, Ulocladium chartarum
     (200.67) and Ulocladium botrytis (452.72).
             Alternatively, Vanadium haloperoxidase can be
30   prepared by recombinant DNA techniques by transforming a
     suitable host by means of an expression vector comprising
     an origin of replication, transcription and termination
     control sequences and the DNA sequence coding for a
     Vanadium haloperoxidase, cultivating the host under
35   conditions which allow the expression of the structural
     gene and isolating the Vanadium haloperoxidase. This is
     described in detail in the Examples below.

(b) The source of halide ions.

      The second constituent of the hygienic
compositions according to the invention is a source of
halide ions. These may be any halide ions, but a source of
5 iodide or chloride ions are preferred because they are most
effective. Sodium chloride is the most preferred source of
halide ions. The halide may be added to the enzymatic
antimicrobial compositions of the invention, or
alternatively, the halide may be used which is naturally
10 present in tap water and which is usually in the order of
2-5 mM.

(c) The source of hydrogen peroxide.

15   The hygienic compositions according to the
invention further comprise a source of hydrogen peroxide,
or a hydrogen peroxide-generating system. Examples of
suitable hydrogen peroxide-generating systems are perborate
or percarbonate salts, preferably sodium percarbonate or
20 sodium perborate.

      The hydrogen peroxide may also be provided by an
enzymatic hydrogen peroxide generating system. The
enzymatic hydrogen peroxide-generating system may in
principle be chosen from the various enzymatic hydrogen
25 peroxide-generating systems which have been disclosed in
the art. For example, one may use an amine oxidase and an
amine, an amino acid oxidase and an amino acid, lactate
oxidase and lactate, cholesterol oxidase and cholesterol,
uric acid oxidase and uric acid or a xanthine oxidase with
30 xanthine. Preferred, however, is the combination of glucose
oxidase and glucose.

      The amount of glucose oxidase will depend on its
specific activity and the activity of any residual catalase
that may be present, but by way of example it can be stated
35 generally that the detergent composition according to the
invention will contain from 10 to 1000, preferably from 20
to 500 units glucose oxidase per g or ml of the detergent
composition, a unit of enzyme activity being defined as the

quantity required to convert 1 μmol of substrate per minute
under standard conditions.

5   (d) Other ingredients.

The enzymatic antimicrobial compositions of the
invention generally comprise from 0.01 to 50 % by weight,
preferably 0.1 to 5.0% by weight of one or more surfactants
are wetting agents. Suitable surfactants or detergent-

10   active compounds are soap or non-soap anionics, nonionics,
cationics, amphoteric or zwitterionic compounds. The
surfactant system usually comprises one or more anionic
surfactants and one or more nonionic surfactants. The
surfactant system may additionally contain amphoteric or

15   zwitterionic detergent compounds, but this is not normally
desired owing to their relatively high cost.

In general, the nonionic and anionic surfactants of
the surfactant system may be chosen from the surfactants
described "Surface Active Agents" Vol. 1, by Schwartz &

20   Perry, Interscience 1949, Vol. 2 by Schwartz, Perry &
Berch, Interscience 1958, in the current edition of
"McCutcheon's Emulsifiers and Detergents" published by
Manufacturing Confectioners Company or in
"Tenside-Taschenbuch", H. Stache, 2nd Edn., Carl Hauser

25   Verlag, 1981.

Suitable nonionic detergent compounds which may
be used include, in particular, the reaction products of
compounds having a hydrophobic group and a reactive
hydrogen atom, for example, aliphatic alcohols, acids,

30   amides or alkyl phenols with alkylene oxides, especially
ethylene oxide either alone or with propylene oxide.
Specific nonionic detergent compounds are C_6-C_{22} alkyl
phenol-ethylene oxide condensates, generally 5 to 25 EO,
i.e. 5 to 25 units of ethylene oxide per molecule, and the

35   condensation products of aliphatic C_8-C_{18} primary or
secondary linear or branched alcohols with ethylene oxide,
generally 5 to 40 EO.

Suitable anionic detergent compounds which may be
used are usually wat r-soluble alkali metal salts of
organic sulphates and sulphonates having alkyl radicals
containing from about 8 to about 22 carbon atoms, the term
5   alkyl being used to include the alkyl portion of higher
acyl radicals. Examples of suitable synthetic anionic
detergent compounds are sodium and potassium alkyl
sulphates, especially those obtained by sulphating higher
C_8-C_{18} alcohols, produced for example from tallow or
10   coconut oil, sodium and potassium alkyl C_9-C_{20} benzene
sulphonates, particularly sodium linear secondary alkyl
C_{10}-C_{15} benzene sulphonates; and sodium alkyl glyceryl
ether sulphates, especially those ethers of the higher
alcohols derived from tallow or coconut oil and synthetic
15   alcohols derived from petroleum. The preferred anionic
detergent compounds are sodium C_{11}-C_{15} alkyl benzene
sulphonates and sodium C_{12}-C_{18} alkyl sulphates.

Also applicable are surfactants such as those
described in EP-A-328 177 (Unilever), which show resistance
20   to salting-out, the alkyl polyglycoside surfactants
described in EP-A-070 074, and alkyl monoglycosides.

Preferred surfactant systems are mixtures of
anionic with nonionic detergent active materials, in
particular the groups and examples of anionic and nonionic
25   surfactants pointed out in EP-A-346 995 (Unilever).
Especially preferred is surfactant system which is a
mixture of an alkali metal salt of a C_{16}-C_{18} primary
alcohol sulphate together with a C_{12}-C_{15} primary alcohol 3-
7 EO ethoxylate.

30   The nonionic detergent is preferably present in
amounts greater than 10%, e.g. 25-90% by weight of the
surfactant system. Anionic surfactants can be present for
example in amounts in the range from about 5% to about 40%
by weight of the surfactant system.

35   The enzymatic detergent compositions of present
invention may also comprise other constituents normally
used in antimicrobial compositions, such as thickening
agents. Particularly useful in this respect are the

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combinations of surfactants disclosed in EP-A-314 232
(Unil v r), which provide thickening gels upon dissolution
with water.

　　　　The antimicrobial compositions of the invention
5 may be employed to provide hygiene benefits for hard-
surface cleaning and fabric washing, but also to provide
hygiene and cleaning in industrial/institutional
applications such as in hospitals and for cleaning and
disinfecting medical equipment. Another application is in
10 the dairy industry, for disinfecting milking equipment. The
antimicrobial compositions can also be succesfully used in
deodorants in view of their ability to combat bacteria
which cause malodour.

　　　　The antimicrobial compositions of the invention
15 may be used in the form of powders which are to be
dissolved in water before use, but the can also be
formulated as liquids products or gels. In those product
forms it is important that the production of hypohalite is
not initiated until the composition is used. This can be
20 achieved by physically separating the enzyme and its
substrate, e.g. by encapsulating the enzyme according to
well known techniques.

　　　　When the enzymatic antimicrobial composition is
used, it is diluted 5 to 100 times by addition of water to
25 provide a medium having an effective antimicrobial
activity. It is then brought into contact with the surface
to be disinfected and allowed

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The invention will be further illustrated by means of
the following non-limiting Examples.

5

EXAMPLE 1
Minimal Inhibitory Concentration (MIC) of hypochlorite.

Materials:
10 Bacterial strains used in this example were Escherichia
coli NCTC 900, Pseudomonas aeruginosa ATCC 15442,
Staphylococcus aureus ATCC 13565, Streptococcus faecalis
NCTC 1092 and Listeria innocua ATCC 33090 serotype 6B.
Bacteria were grown for 15 to 18 hours at 30°C in Brain
15 Heart Infusion (BHI) broth-medium. After cultivation the
strains were washed two times in citrate buffer pH 5.5 (20
mM Na$_3$citrate-NaOH buffer + 10 mM NaCl). The bacterium
suspensions were centrifuged in an Eppendorf centrifuge
(14,000 rpm for 5 min), then the supernatants were removed
20 and subsequently the bacterial pellet was resuspended in
citrate buffer. This washing procedure was then repeated
once more for each bacterial suspension. The twice washed
bacterial suspension was then diluted with citrate buffer
pH 5.5 to obtain a suspension of approximately 10^7 bacteria
25 per ml. During the washing steps the cells were kept on
ice. Buffers and BSA-solution (1% w/v in citrate buffer pH
5.5) were filter-sterilised and stored at 4°C. The
hypochlorite solutions were made from a stock-solution
(107,000 ppm) by dilution with sterile demineralised water.
30

Methodology:
Using sterile techniques, a suspension of approximately 10^7
bacteria per ml in citrate buffer pH 5.5 was made. From
this suspension aliquots of 1.9 ml were added to sterile
35 tubes. Subsequently 0.1 ml of cold hypochlorite solutions
of various concentrations were added to the tubes, in such
a way that a hypochlorite dilution range is obtained. The
tubes were mixed continuously with a magnetic stirrer. Also

blank determinations were included where only sterile
buffer was added instead of a hypochlorite solution. The
samples were incubated for exactly 5 min at 30°C. After
this incubation period 1 ml of the reaction mixture was

5   taken and added to 9 ml cold BSA (1% w/v) solution and put
on ice immediately. This was done to stop the reaction of
hypochlorite with the microorganisms. Survival as Colony
Forming Units (CFU) per ml was determined by diluting the
sample from 10^{-1} to 10^{-5} and plating 100 μl samples of the

10 various dilutions on labelled BHI-agar plates. The plates
were then incubated for 15-18 hours at 30°C. When there
were no CFU detectable after this incubation period, the
plates are incubated for another 24 hours at 30°C.

15 Definition: the Minimal Inhibitory Concentration (MIC-
value) is defined here as the concentration of hypochlorite
which leads under the experimental conditions used to at
least a log 6 reduction in colony forming units of the
specific microorganism tested.

20

The results obtained are shown in Table I. The values found
are in the same range as reported in the literature.

Table I. MIC values for various microorganisms.

Microorganism	MIC-value in ppm
Escherichia coli	2-3
Staphylococcus aureus	2-3
Listeria innocua	2-3
Pseudomonas aeruginosa	2-3
Streptococcus faecalis	2-3

EXAMPLE 2
Minimal Inhibitory Concentration of hypochlorite and
hypochlorite generated enzymatically by vanadium-
chloroperoxidase (V-CPO) from Curvularia inaequalis.

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In this example the killing effect of hypochlorite is
compared to the killing effect of hypochlorite generated
enzymatically by vanadium-chloroperoxidase (V-CPO) from
Curvularia inaequalis. In order to make comparisons
10 possible, a micropump was used. This was done to make the
hypochlorite concentration during the experiment follow the
same profile as in the situation were hypochlorite is
generated enzymatically. Also the V-CPO activity was
determined very carefully under the conditions of the
15 experiment, this to know the amount of hypochlorite present
at each time point of the experiment.

Materials:
Bacterial strains used in this example were Escherichia
20 coli NCTC 900, Pseudomonas aeruginosa ATCC 15442,
Staphylococcus aureus ATCC 13565, Streptococcus faecalis
NCTC 1092 and Listeria innocua ATCC 33090 serotype 6B.
Bacteria were grown for 15 to 18 hours at 30°C in Brain
Heart Infusion (BHI) broth-medium. After cultivation the
25 strains were washed two times in citrate buffer pH 5.5 (20
mM Na_3citrate-NaOH buffer + 10 mM NaCl). The bacterium
suspensions were centrifuged in an Eppendorf centrifuge
(14000 rpm for 5 min), then the supernatant were removed
and subsequently the bacterial pellet was resuspended in
30 citrate buffer. This washing procedure was then repeated
once more for each bacterial suspension. The twice washed
bacterial suspension was then diluted with citrate buffer
pH 5.5 to obtain a suspension of approximately 10^7 bacteria
per ml. During the washing steps the cells were kept on
35 ice. Buffers and BSA-solution (1% w/v in citrate buffer pH
5.5) were filter-sterilised and stored at 4°C. The
hypochlorite solutions were made from a stock-solution
(107,000 ppm) by dilution with sterile demineralised water.

H_2O_2 solutions were made from a 30% stock solution by dilution with sterile demineralised water. Casitone was obtained from Difco. Chloroperoxidase from _Curvularia inaequalis_ was purified according to van Schijdel et al.

5 (1993). Chloroperoxidase activity in the conversion of Cl- to HOCl was determined spectrophotometrically in 20 mM sodium citrate buffer pH 5.5, 10 mM NaCl, 100 μM H_2O_2, 50 μM monochlorodimedone at 30°C, by following the conversion of monochlorodimedone (e 290 nm = 20.2 $mM^{-1}.cm^{-1}$) to

10 dichlorodimedone (e 290 nm = 0.2 $mM^{-1}.cm^{-1}$). 1 Unit of chloroperoxidase is defined as the amount of enzyme that converts 1 μmol of monochlorodimedone (Sigma) per minute.

Methodology:

15 Using sterile techniques, a suspension of approximately 10^7 bacteria per ml in citrate buffer pH 5.5 was made. From this suspension aliquots of 1.8 ml were added to a sterile reaction vessel, which was continuously stirred throughout the experiment with a magnetic stirrer. Subsequently 0.2 ml

20 of V-CPO solutions of various concentrations (for calculations, see below) were added to the reaction vessels, in such a way that a V-CPO dilution range is obtained. Also blank determinations were included where 0.2 ml sterile buffer was added instead 0.2 ml of V-CPO

25 solution. The reaction vessels was incubated at 30°C. Then 0.5 ml of a H_2O_2 stock solution was added, to start the V-CPO reaction. The H_2O_2 stockconcentration used depended on the hypochlorite concentration produced, and was chosen in such a way that the endconcentration of H_2O_2 (at the end of

30 the flux) was a fivefold molar excess compared to the endconcentration of hypochlorite. The samples were incubated for exactly 5 min at 30°C. After this incubation period 1 ml of the reaction mixture was taken and added to 9 ml cold BSA (1% w/v) solution and put on ice immediately.

35 This was done to stop the reaction of hypochlorite with the microorganisms. Survival as Colony Forming Units (CFU) per ml was determined by diluting the sample from 10^{-1} to 10^{-5} and plating 100 μl samples of the various dilutions on

labelled BHI-agar plates. The plates were then incubated
for 15-18 hours at 30°C. When there were no CFU detectable
after this incubation period, the plates are incubated for
another 24 hours at 30°C.

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The MIC value obtained was compared to the MIC value that
was obtained when the same hypochlorite amounts were added,
using a micropump. This was done as follows:

10 Using sterile techniques, a suspension of approximately 10^7
bacteria per ml in citrate buffer pH 5.5 was made. From
this suspension aliquots of 1.8 ml were added to a sterile
reaction vessel, which was continuously stirred throughout
the experiment with a magnetic stirrer. The reaction
15 vessels were incubated at 30°C. Then 0.2 ml of a H_2O_2
stock solution was added. The H_2O_2 stock concentration used
depended on the hypochlorite concentration produced, and
was chosen in such a way that the end concentration of H_2O_2
(at the end of the flux) was a five-fold molar excess
20 compared to the endconcentration of hypochlorite. Then a
flux of 0.5 ml of a hypochlorite solutions of a known
concentration was applied over 5 min (flow: 0.1 ml per min)
at 30°C. A series of experiments were done, each using
various hypochlorite solutions of a different
25 concentration, so that a range of hypochlorite end
concentrations was obtained (for calculations see below).
After the incubation period of 5 minutes, 1 ml of the
reaction mixture was taken and added to 9 ml cold BSA (1%
w/v) solution and put on ice immediately. This was done to
30 stop the reaction of hypochlorite with the microorganisms.
Survival as Colony Forming Units (CFU) per ml was
determined by diluting the sample from 10^{-1} to 10^{-5} and
plating 100 μl samples of the various dilutions on labelled
BHI-agar plates. The plates were then incubated for 15-18
35 hours at 30°C. When there were no CFU detectable after this
incubation period, the plates are incubated for another 24
hours at 30°C.

The similarity of the hypochlorite profiles obtained in the
experiments described above with V-CPO respectively with
hypochlorite from a stock solution were confirmed by using
the same experimental set-up (as for the blank experiments

5 without the microorganisms added) coupled to a
spectrophotometer, in which the hypochlorite concentrations
in time could be followed at 290 nm, making use of the
conversion of monochlorodimedone (e 290nm = 20.2 $mM^{-1}.cm^{-1}$)
to dichlorodimedone (e 290nm = 0.2 $mM^{-1}.cm^{-1}$).

10

Calculations:
The endconcentration of hypochlorite generated with V-CPO
was calculated as follows:
With 0.01 U per ml of V-CPO 0.01 μmol of hypochlorite per

15 ml per min is generated, which is equivalent to 0.05 μmol
hypochlorite per ml per 5 min. due to the dilution effect
of the flux of 0.5 ml, which is added to 2.0 ml initial
volume, the endconcentration after 5 minutes is (2.0/2.5) *
0.05 μmol per ml = 0.04 μmol hypochlorite per ml after 5

20 minutes. This concentration could subsequently be expressed
as 0.04 μmol hypochlorite per ml = 0.04 * 52.5 ppm
hypochlorite = 2.10 ppm hypochlorite. Other hypochlorite
endconcentrations were obtained by increasing or decreasing
the V-CPO concentration. The endconcentration of

25 hypochlorite added was calculated as follows:
Since a flux of 0.5 ml is added to a reaction volume of 2.0
ml, the endvolume is 2.5 ml, which corresponds to diluting
fivefold. In order to obtain a hypochlorite end
concentration of 2.10 ppm, a stock solution of 10.50 ppm

30 was used. Other hypochlorite concentrations were obtained
by increasing or decreasing the hypochlorite stock
solution.

Definition: the Minimal Inhibitory Concentration (MIC-

35 value) is defined here as the concentration of hypochlorite
which leads under the experimental conditions used to at
least a log 6 reduction in colony forming units of the
specific microorganism tested.

17

·The results are shown in Table II. The killing effects of
various endconcentrations of hypochlorite, either produced
by V-CPO or at the end of- the hypochlorite flux, are
compared. As can be concluded from Table II, hypochlorite
5 produced enzymatically by V-CPO already gives total
inhibition of growth at hypochlorite concentrations as low
as 0.4 ppm for all organisms tested except for
Staphylococcus aureus, whereas 2 ppm hypochlorite is needed·
to obtain the same growth inhibition. This shows that
10 vanadium-containing haloperoxidase is a more efficient
hygienic component than would have been expected from just
its hypochlorite producing capacity. Also it is apparent
that V-CPO gives killing of all microorganisms tested,
pathogenic or non-pathogenic, whereas heme-containing
15 haloperoxidase are claimed to give only efficient killing
of pathogenic bacteria (see EP-A-500 387).

Table II. MIC values for various microorganisms.

Microorganism	MIC-value with hypochlorite (in ppm)	MIC-value with V-CPO (in ppm)
Escherichia coli	2	0.4
Staphylococcus aureus	2-3	1.6
Listeria innocua	2	0.4
Pseudomonas aeruginosa	2	0.4
Streptococcus faecalis	2	0.4

EXAMPLE 3
Killing efficiencies of hypochlorite and hypochlorite·
generated enzymatically by V-CPO in the presence of protein
30 hydrolysate.

For obtaining hygiene it is known that in situations
encounter d in practice, overdosing of hypochlorite is
necessary, since this reactive molecule will not only react
with microorganisms, but also with other compounds that are
5 present. Therefor it was important to test the behaviour of
vanadium-containing haloperoxidase in a fluid containing
e.g. a protein hydrolysate.

Materials:
10 The bacterial strain used in this example was _Escherichia_
coli NCTC 900. Bacteria were grown for 15 to 18 hours at
30°C in Brain Heart Infusion (BHI) broth-medium. After
cultivation the strains were washed two times in citrate
buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10 mM NaCl).
15 The bacterium suspensions were centrifuged in an Eppendorf
centrifuge (14,000 rpm for 5 min), then the supernatants
were removed and subsequently the bacterial pellet was
resuspended in citrate buffer. This washing procedure was
then repeated once more for each bacterial suspension. The
20 twice washed bacterial suspension was then diluted with
citrate buffer pH 5.5 to obtain a suspension of
approximately 10^8 bacteria per ml. During the washing steps
the cells were kept on ice. Buffers and BSA-solution (1%
w/v in citrate buffer pH 5.5) were filter-sterilised and
25 stored at 4°C. The hypochlorite solutions were made from a
stock-solution (107,000 ppm) by dilution with sterile demi.
H_2O_2 solutions were made from a 30% stock solution by
dilution with sterile demineralised water. Casitone was
obtained from Difco. Chloroperoxidase from _Curvularia_
30 _inaequalis_ was isolated and purified according to Van
Schijndel et al., 1993.

Methodology:
Using sterile techniques, a suspension of approximately 10^8
35 bacteria per ml in citrate buffer pH 5.5 was made. From
this suspension aliquots of 1.3 ml were added to a sterile
reaction vessel, which was continuously stirred throughout
the experiment with a magnetic stirrer. Then 0.5 ml of a

0.5 mg per ml casitone (in citrate buffer pH 5.5) solution,
respectively 0.5 ml citrate buffer pH 5.5 were added.
Subsequently 0.2 ml of V-CPO solutions of various
concentrations (for calculations see Example 2) were added
5   to the reaction vessels, in such a way that end
concentrations of 3.2 ppm hypochlorite or 6.5 ppm
hypochlorite were achieved. Also blank determinations were
included were 0.2 ml sterile buffer was added instead 0.2
ml of a V-CPO solution. The reaction vessels were incubated
10   at 30°C. Then 0.5 ml of a H_2O_2 solution was added (the
concentration was chosen so that a fivefold molar excess of
hydrogen peroxide was obtained at the end of the flux), to
start the V-CPO reaction. The samples were incubated for
exactly 5 min at 30°C. After this incubation period 1 ml of
15   the reaction mixture was taken and added to 9 ml cold BSA
(1% w/v) solution and put on ice immediately. This was done
to stop the reaction of hypochlorite with the
microorganisms. Survival as Colony Forming Units (CFU) per
ml was determined by diluting the sample from 10^{-1} to 10^{-6}
20   and plating 100 μl samples of the various dilutions on
labelled BHI-agar plates. The plates were then incubated
for 15-18 hours at 30°C. When there were no CFU detectable
after this incubation period, the plates are incubated for
another 24 hours at 30°C.
25

The killing efficiencies obtained was compared to the
killing efficiencies obtained when the same hypochlorite
amounts were added, using a micropump. This was done as
follows:
30

Using sterile techniques, a suspension of approximately 10^8
bacteria per ml in citrate buffer pH 5.5 was made. From
this suspension aliquots of 1.3 ml were added to a sterile
reaction vessel, which was continuously stirred throughout
35   the experiment with a magnetic stirrer. Then 0.5 ml of a
0.5 mg per ml casitone solution (in citrate buffer pH 5.5),
respectively 0.5 ml citrate buffer pH 5.5 were added.
Subsequently 0.2 ml of sterile citrate buffer pH 5.5 was

added. The reaction vessels were incubated at 30°C. Then a
flux of 0.5 ml of hypochlorite solutions, yielding an
endconcentration of 3.2 ppm hypochlorite respectively 6.5
ppm hypochlorite (for calculations on the concentration
5   see Example 2) were applied over 5 min (flow: 0.1 ml per
min) at 30°C. After the incubation period of 5 min 1 ml of
the reaction mixture was taken and added to 9 ml cold BSA
(1% w/v) solution and put on ice immediately. This was done
to stop the reaction of hypochlorite with the
10   microorganisms. Survival as Colony Forming Units (CFU) per
ml was determined by diluting the sample from 10^{-1} to 10^{-6}
and plating 100 μl samples of the various dilutions on
labelled BHI-agar plates. The plates were then incubated
for 15-18 hours at 30°C. When there were no CFU detectable
15   after this incubation period, the plates are incubated for
another 24 hours at 30°C. The results are shown in Table
III.

Table III. The influence of casitone on the killing
20   efficiencies of HClO, generated by V-CPO.

casitone concentration (mg/ml)	log reduction with 3.2 ppm hypochlorite generated by V-CPO	log reduction with 3.2 ppm hypochlorite	log reduction with 6.5 ppm hypochlorite generated by V-CPO	log reduction with 6.5 ppm hypochlorite
0.0	8.0 (= total kill)	8.2 (= total kill)	8.0 (= total kill)	8.2 (= total kill)
0.1	5.0	0.5	8.0 (= total kill)	1.0

EXAMPLE 4
Comparison between the killing efficiencies of Vanadium
containing haloperoxidase and a heme-containing
haloperoxidase.

5

Materials:
Bacterial strains used in this example were Escherichia
coli NCTC 900, Streptococcus faecalis NCTC 1092 and
Listeria innocua ATCC 33090 serotype 6B. Bacteria were
10   grown for 15 to 18 hours at 30°C in Brain Heart Infusion
(BHI) broth-medium. After cultivation the strains were
washed two times in citrate buffer pH 5.5 (20 mM
Na_3citrate-NaOH buffer + 10 mM NaCl). The bacterium
suspensions were centrifuged in an Eppendorf centrifuge
15   (14,000 rpm for 5 min), then the supernatants were removed
and subsequently the bacterial pellet was resuspended in
citrate buffer. This washing procedure was then repeated
once more for each bacterial suspension. The twice washed
bacterial suspension was then diluted with citrate buffer
20   pH 5.5 to obtain a suspension of approximately 10^7 bacteria
per ml. During the washing steps the cells were kept on
ice. Buffers and BSA-solution (1% w/v in citrate buffer pH
5.5) were filter-sterilised and stored at 4°C.
Chloroperoxidase from Curvularia inaequalis was purified
25   according to van Schijndel et al., (1993). The heme-
containing chloroperoxidase was obtained from Sigma (ex
Caldariomyces fumago). Chloroperoxidase activity in the
conversion of Cl⁻ to HOCl was determined
spectrophotometrically in 20 mM sodium citrate buffer pH
30   5.5, 10 mM NaCl, 100 μM H_2O_2, 50 μM monochlorodimedone at
30°C, by following the conversion of monochlorodimedone (e
290 nm = 20.2 $mM^{-1}.cm^{-1}$) to dichlorodimedone (e 290 nm =
0.2 $mM^{-1}.cm^{-1}$). 1 Unit of chloroperoxidase is defined as
the amount of enzyme that converts 1 μmol of
35   monochlorodimedone (Sigma) per minute. The same assay (and
activity definition) was used for both enzymes, in order to
be able to dose the two enzymes at equeal activity.

Methodology:
Using sterile techniques a suspension of approximately 10^7
bacteria per ml in 20 mM sodium citrate buffer pH 5.5, 10
mM NaCl was made. From this suspension aliquots of 1.9 ml
5   were added to sterile tubes. Subsequently 0.1 ml of stock
solutions of vanadium-containing chloroperoxidase
respectively of heme-containing chloroperoxidase were added
to the tubes, in such a way that a dilution range for both
enzymes was obtained. Also blank determinations were
10   included where only sterile buffer was added instead of an
enzyme stock solution. Then 0.5 ml of an 25 mM H_2O_2 stock
solution was added. The sample were incubated for exactly 5
min at 30°C. After this incubation period 1 ml of the
reaction mixture was taken and added to 9 ml cold BSA (1%
15   w/v) solution and put on ice immediately. Survival as
Colony Forming Units (CFU) per ml was determined by
diluting the sample from 10^{-1} to 10^{-5} and plating 100 μl
samples of the various dilutions on labelled BHI-agar
plates. The plates were then incubated for 15-18 hours at
20   30°C. When there were no CFU detectable after this
incubation period, the plates are incubated for another 24
hours at 30°C.

The results are presented in Table IV. The data clearly
25   show that vanadium-containing chloroperoxidase provides a
by far more effective hygienic system than the heme-
containing chloroperoxidase, even when they are dosed at
equal activity.

30

Table IV.

10 mM NaCl	vanadium chloro-peroxidase (U per ml)	heme chloro-peroxidase (U per ml)	log reduction
E coli	0.000		0.0
	0.00156		4.0
	0.0030		5.0
	0.0060		7.0(=total kill)
	0.012		7.0(=total kill)
	0.025		7.0(=total kill)
	0.050		7.0(=total kill)
	0.10		7.0(=total kill)
		0.000	0.0
		0.00156	0.1
		0.0030	0.1
		0.0060	0.1
		0.012	0.2
		0.025	1.0
		0.050	1.2
		0.10	1.2

L. innocua	0.000		0.0
	0.00156		7.0 (total kill)
	0.0030		7.0 (total kill)
	0.0060		7.0 (total kill)
	0.012		7.0 (total kill)
	0.025		7.0(total kill)
	0.050		7.0(total kill)
	0.10		7.0(total kill)
		0.00	0.0
		0.00156	0.0
		0.0030	0.0
		0.0060	0.0
		0.012	0.0
		0.025	0.0
		0.050	0.0
		0.10	0.0
S.faecalis	0.00		0.0
	0.00156		5.0
	0.0030		5.0
	0.0060		7.0(total kill)
	0.012		7.0(total kill)
	0.025		7.0 (total kill)
	0.050		7.0(total kill)
	0.10		7.0(total kill)
		0.00	0.0
		0.00156	0.0
		0.0030	0.1
		0.0060	0.1
		0.012	0.1
		0.025	0.1
		0.050	0.1
		0.10	0.9

EXAMPLE 5

The effect of various hydrogen peroxide sources on the
inhibitory action of V-CPO.

5   In the previous examples hydrogen peroxide, which is one of
     the substrates in the V-CPO reaction, was added from a
     stock solution. In this example the effects of using other
     hydrogenperoxide sources is described. Oxidases were
     assayed at 30°C in a Biological Oxygen Monitor YSI Model
10   5300 (Oxygen chamber model 5301) in which oxygen
     consumption was monitored, using citrate buffer pH 5.5 (20
     mM Na_3citrate-NaOH buffer + 10 mM NaCl) as a buffer. The
     Buffer were saturated with air at 30°C. As a substrate for
     glucose oxidases 15 g.l^{-1} (end concentration) glucose was
15   used. The bacterial strain used in this example was
     Escherichia coli NCTC 900. Bacteria were grown for 15 to
     18 hours at 30°C in Brain Heart Infusion (BHI) broth-
     medium. After cultivation the strains were washed two times
     in citrate buffer pH 5.5 (20 mM Na_3citrate-NaOH buffer + 10
20   mM NaCl). The bacterium suspensions were centrifuged in an
     Eppendorf centrifuge (14,000 rpm for 5 min), then the
     supernatant were removed and subsequently, the bacterial
     pellet was resuspended in citrate buffer. This washing
     procedure was then repeated once more for each bacterial
25   suspension. The twice washed bacterial suspension was then
     diluted with citrate buffer pH 5.5 to obtain a suspension
     of approximately 10^8 bacteria per ml. During the washing
     steps the cells were kept on ice. Buffers and BSA-solution
     (1% w/v in citrate buffer pH 5.5) were filter-sterilised
30   and stored at 4°C. The hypochlorite solutions were made
     from a stock-solution (107,000 ppm) by dilution with
     sterile demi. H_2O_2 solutions were made from a 30% stock
     solution by dilution with sterile demineralised water.
     Casitone was obtained from Difco. Chloroperoxidase from
35   Curvularia inaequalis was isolated and purified according
     to Van Schijndel et al.,(1993). Glucose oxidase ex
     Aspergillus niger was obtained from Sigma.

Methodology:
Using sterile techniques, a suspension of approximately 10^8
bacteria per ml in citrate buffer pH 5.5 was made. From
this suspension aliquots of 1.3 ml were added to a sterile
5   reaction vessel, which was continuously stirred throughout
the experiment with a magnetic stirrer. Then 0.5 ml of a
0.5 mg per ml casitone solution (in citrate buffer pH 5.5)
was added. Subsequently, 0.2 ml of V-CPO solutions were
added to the reaction vessels to give a final concentration
10. of 6.5 ppm hypochlorite. Also blank determinations were
included were 0.2 ml sterile buffer was added instead 0.2
ml of a V-CPO solution. The reaction vessels was incubated
at 30°C. Then 0.5 ml of one following three hydrogen
peroxide generating systems were added:
15   1. H_2O_2 from a 3 mM stock solution;
      2.   sodium percarbonate from a 3 mM stock solution;
      3.   a mixture of glucose oxidase (0.39 Units/ml) and 75
mg/ml glucose.

20   The samples were incubated for exactly 5 min at 30°C. After
this incubation period 1 ml of the reaction mixture was
taken and added to 9 ml cold BSA (1% w/v) solution and put
on ice immediately. This was done to stop the reaction of
hypochlorite with the microorganisms. Survival as Colony
25   Forming Units (CFU) per ml was determined by diluting the
sample from 10^{-1} to 10^{-6} and plating 100 μl samples of the
various dilutions on labelled BHI-agar plates. The plates
were then incubated for 15-18 hours at 30°C. When there
were no CFU detectable after this incubation period, the
30   plates are incubated for another 24 hours at 30°C. The
values obtained were compared to the MIC value that was
obtained when exactly the same amounts of hypochlorite were
added, using a micropump. This was done as follows:

35   Using sterile techniques, a suspension of approximately 10^8
bacteria per ml in citrate buffer pH 5.5 was made. From
this suspension aliquots of 1.3 ml were added to a sterile
reaction vessel, which was continuously stirred throughout

27

the experiment with a magnetic stirrer. Then 0.5 ml of a
casitone solution (0.5 mg/ml in citrate buffer pH 5.5) was
added. Subsequently 0.2 ml of sterile citrate buffer pH
5.5 was. The reaction vessels was incubated at 30°C. Then a
5   flux of 0.5 ml of a hypochlorite solutions of 32.5 ppm was
applied over 5 min (flow: 0.1 ml per min) at 30°C. After
the incubation period of 5 min 1 ml of the reaction mixture
was taken and added to 9 ml cold BSA (1% w/v) solution and
put on ice immediately. This was done to stop the reaction
10 of hypochlorite with the microorganisms. Survival as Colony
Forming Units (CFU) per ml was determined by diluting the
sample from 10^{-1} to 10^{-6} and plating 100 μl samples of the
various dilutions on labelled BHI-agar plates. The plates
were then incubated for 15-18 hours at 30°C. When there
15 were no CFU detectable after this incubation period, the
plates are incubated for another 24 hours at 30°C. The
results are shown in Table V.

Table V. V-CPO reaction with different H_2O_2 sources.

hydrogen-peroxide source	casitone concen-tration (mg/ml)	log reduction at 6.5 ppm hypo-chlorite	log reduction at 6.5 ppm hypochlorite generated enzymatically by V-CPO
H_2O_2 stock solution	0.0	8.2 (= total kill)	8.0 (= total kill)
per-carbonate	0.0	8.0 (= total kill)	8.2 (=total kill)
glucose oxidase	0.0	6.4 (= total kill)	6.4 (= total kill)
H_2O_2 stock solution	0.1	1.0	8.0 (= total kill)
per-carbonate	0.1	0.8	8.2 (= total kill)
glucose oxidase	0.1	1.0	6.4 (= total kill)

EXAMPLE 6

Method to determine the coding sequence of the chloro
peroxidase gene (cDNA) and the gene from Curvularia
inaequalis (Centraal Bureau voor Schimmelcultures, the
Netherlands, strain No 102.42) and possible expression
systems.

Chloroperoxidase was isolated and purified from liquid
cultures of C. inaequalis as described by Van Schijndel et

al., 1993), except that after DEAE chromatography two
additional purification steps were performed using an FPLC
system (Pharmacia LKB). First a phenyl-sepharose Cl-4B
hydrophobic interaction column was used to bind the enzyme
5   in the presence of 2 M NaCl in 50 mM Tris-HCl (pH 8.3),
followed by elution with a descending gradient from 2 M
NaCl in 50 mM Tris-HCl (pH 8.3). For the final purification
a MonoQ HR 5/5 anion exchange column (ex Pharmacia LKB) was
used to bind the enzyme, followed by elution with a
10   gradient from 0 M to 0.5 M NaCl in 20 mM piperazine-HCl (pH
5.4). Subsequent concentration of the enzyme was carried
out using rotation evaporation, followed by dialysis
against 50 mM Tris-SO$_4$ buffer (pH 8). The purified
chloroperoxidase was enzymatically digested with the
15   proteases Staphylococcus V8 and trypsin respectively,
according to standard procedures known in the art, or
chemically cleaved with CNBr (Gross, E. (1967), Methods
Enzymology 11, 238-255). The resulting peptides were
separated using SDS-PAGE according to Laemmli (Laemmli,
20   U.K. (1970) Nature 227, 680-685) or on a tricine gel as
according to Schagger and Von Jagow (1987) and subsequently
transferred to PVDF membranes (Immobilon-P ex Millipore)
using CAPS transfer buffer (10 mM 3-[cyclohexylamino]-1-
propanesulfonic acid, 10% methanol, pH 11) as described by
25   Matsudaira (1987). After electrophoretic elution the
membrane was rinsed for 5 min with deionized water, stained
with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min,
and destained in 50% methanol, 10% acetic acid for 10 min
at room temperature and air dried. Peptide bands were
30   submitted to automatic Edmann sequencing on a Porton LF
3000 protein sequencer (Beckman Instruments, Inc., USA).
The results of the amino acid sequence determination are
summarized in Figure 1.

35   Based on the amino acid sequences of the peptides fully
degenerated oligonucleotides were designed (see Table VII).
These degenerated primers were used in Polymerase Chain

Reaction (PCR) reactions, using first-strand cDNA as a
template. First-strand cDNA was prepared as follows:
For the isolation of RNA spores of C. inaequalis were
inoculated into a fermentation medium containing 4 g yeast
5   extract and 2 ml of a microelement solution (Van Schijndel
et al., 1993) per litre. After several days of growth the
mycelium were harvested by filtration and lyophilized. The
lyophilized C. inaequalis mycelia were ground under liquid
nitrogen. RNA was extracted by adding an RNA extraction
10 buffer (42 mM sodium citrate pH 7, 0.83% N-laurylsarcosine,
50 mM beta-mercaptoethanol, 1% Triton X-100 and 4 M
guanidine isothiocyanate), and incubating for 1 hour at
room temperature. 0.1 Volumes of 2 M sodium acetate (pH 4)
and 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1)
15 was added and the mixture placed on ice for 15 min. After
centrifugation for 10 min at 10000xg (4°C), the aqueous
phase was collected, 1 volume of absolute alcohol was added
and the mixture was incubated for 1 hour at -20°C followed
by brief centrifugation at 10,000xg. The pellet was
20 resuspended in an appropriate volume of RNA extraction
buffer and fractionated by ultracetrifugation in a gradient
of cesium chloride (Sambrook et al., 1989). The pellet was
carefully washed and stored in a 75% ethanol solution at -
70°C. For mRNA isolation the RNA was precipitated and
25 resuspended in RNAse free water after which the mRNA was
extracted using the polyAtract mRNA isolation kit (Promega
corporation, USA). First strand cDNA synthesis was carried
out on isolated mRNA from C. inaequalis using the Pharmacia
first-strand cDNA synthesis kit (Pharmacia Biotech). Four
30 20-meric degenerated oligonucleotides were designed based
on the amino acid sequences of chloroperoxidase peptides
(see also Table VII) and used as primers in polymerase
chain reactions with first strand cDNA from C. ineaqualis
as a template. Polymerase chain reactions were performed
35 using a thermocycler (Eppendorf mastercycler 5330) and Taq
polymerase (Promega corporation). For optimal amplification
of the chloroperoxidase encoding cDNA using the degenerated
primers the polymerase chain reaction was performed at 46°C

(annealing step) for 30 cycles. The two resulting specific
fragments were ligated into a pUC18 vector, cloned and
sequenced from both strands. Based on the DNA sequence
results the following two specific primers were designed:

5   5'- CATAGCGATAGCGACGCGGA-3'

        and

10   5'- CTAACCCCGGCGCCAACATC-3'

        These two primers were used in polymerase chain reactions
        with first strand cDNA as a template. Thus a gene-specific
        DNA fragment was obtained joining the two known DNA
15   sequences. This fragment was cloned in a pUC18 vector and
        subsequently sequenced. To obtain the 5' region of the mRNA
        encoding the chloroperoxidase the 5'-Amplifinder RACE kit
        (Clonetech corporation) was used. The genomic
        chloroperoxidase gene from C. ineaqualis was isolated as
20   follows:
        C. ineaqualis genomic DNA was isolated from lyophilized
        mycelia which was ground under liquid nitrogen and
        extracted with an appropriate amount of extraction buffer
        (200 mM Tris-HCl, pH 8.5, 25 mM EDTA, 250 mM NaCl, 1% SDS
25   and 0.2 mg per ml proteinase K). After incubation overnight
        at room temperature 0.7 volumes of phenol and 0.3 volumes
        of chloroform were added and mixed vigorously. The tubes
        were centrifuged at 10,000xg and the aqueous layer was
        transferred to a clean tube. The genomic DNA was
30   precipitated with 2 volumes of absolute ethanol. After
        centrifugation for 5 min at 5000xg the pellet was
        resuspended in 2 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA
        and treated with RNAse (Boehringer Mannheim) as recommended
        by the manufacturer. The genomic DNA containing solution
35   was extracted with phenol:chloroform:isoamylalcohol
        (25:24:1) and after ethanol precipitation, finally
        dissolved in a suitable volume of 10 mM Tris-HCl pH 8, 1 mM
        EDTA buffer. For Southern analysis of the genomic DNA, the

DNA was digested with several combinations of restriction
enzymes and after agarose gel electrophoresis blotted to a
nitrocellulose membrane (Sambrook et al., 1989).
Hybridization was carried out using a radiolabeled gene
5   specific fragment (amplified with the two specific primers
described above), which was made by random priming using
alpha-32P labelled dATP (Sambrook et al., 1989). Based on
the results obtained a mini-library was made using genomic
DNA digested with Pst I which was inserted in the vector
10 pUC18. The library was screened with the same probe as
described for the Southern blot. A positive clone was
isolated and also partly sequenced from both strands to
confirm the cDNA sequence results. The C. inaequalis
chloroperoxidase encoding gene and its putative gene
15 product are disclosed in Figure 2.
A chloroperoxidase production system in Saccharomyces
cerevisiae was made as follows:
The well known GAL1 inducible yeast promoter was obtained
as an EcoR1 BamH1 fragment from the S. cerevisiae wild type
20 GAL1 gene (Molecular and Cellular Biology 10, 4757-4769,
1990) and cloned into the EcoR1 BamH1 sites of respectively
plasmid YCplac33 and YEplac95 (Gietz and Sugino (1988) Gene
74, 527-534). The plasmids obtained were named TNT1
respectively TNT2. A BamH1 restriction site was created in
25 front of the 5' begin of the C. inaequalis chloroperoxidase
gene by performing a PCR experiment using as a template the
PstI EcoRI 5' fragment of the C. inaequalis
chloroperoxidase gene subcloned into pUC18 and as primers
the M13/pUC 22-mer reverse sequence primer and the primer :
30
5' GAG AGA GGA TCC ACT CAC TAC TTA CAA TCA CAC 3'

The amplified fragment was digested with BamHI and EcoRI.
The EcoRI PvuII fragment from the C. inaequalis
35 chloroperoxidase gene, containing the 3' part of the gene,
was subcloned into EcoRI SmaI digested pUC18. From this
clone, after digesting it with EcoRI and XbaI, a fragment

33

containing the 3' part of the C. inaequalis chloro-
peroxidase gene was purified.
A three point ligation was performed, which comprised
either TNT1 or TNT2 digested each with BamHI and XbaI, and
5   the 5' BamHI EcoRI fragment and the 3' EcoRI XbaI fragment.
After ligation and cloning the obtained plasmids were
checked for their identity. Plasmids thus obtained were
named TNT3 (derived from TNT1) respectively TNT4 (derived
from TNT2).
10 Yeast strain BJ1991 was transformed with plasmids TNT3
respectively TNT4 according to procedures known in the art
and selected for ura+ transformants. Ura+ transformants
were replicated to YP-plates containing either glucose (2%)
or galactose (2%). After growth some cells were taken from
15 the plates and resuspended in 200 μl 20 mM Tris-HCl pH 8.1.
After incubation for 5 minutes 10 μl was taken from the
fluid and spotted on a nitrocellulose filter. The
nitrocellulose filters were incubated in 100 mM sodium
acetate buffer (pH 5.5), 1 mM orthodianisidine, 100 mM KBr
20 and 2 mM H_2O_2. A clear colour formation was observed on all
spots derived from galactose grown yeast strains, whereas
no colour formation was observed for the glucose grown
yeasts. This indicates that a galactose inducible
production system for the Curvularia inaequalis
25 chloroperoxidase gene in the yeast Saccharomyces cerevisiae
has been constructed. A similar assay, making use of 100 mM
potassium phosphate buffer (pH 6.5), 100 mM KBr, 1 mM H_2O_2
and 40 μM phenol red (BDH), clearly showed a blue/purple
colour formation with fluids from galactose grown yeast,
30 whereas no colour changes occurred with fluids from glucose
grown yeasts. To further confirm that a heterologous
chloroperoxidase gene expression system in yeast was made
that produced C. inaequalis chloroperxidase with the same
functionality as the C. inaequalis chloroperoxidase, the
35 recombinant enzyme was purified from galactose induced TNT3
or TNT4 transformed yeast strains. After growth in
galactose containing medium, yeast cells were harvested and

resuspended in 20 mM Tris-HCl (pH 8.1). Sterile glass beads
were then added and the suspension was shaken vigorously.
After centrifugation for 15 minutes at 10,000xg the
supernatant was taken and applied to a DEAE column as the
5   recombinant enzyme was purified using essentially the
purification protocol as for the wild type $\underline{C.\ inaequalis}$
enzyme (see above). After purification a recombinant
chloroperoxidase was obtained with a specific activity of
22 U per mg protein (determined in 100 mM sodium acetate
10 buffer pH 5.0, 1 mM H_2O_2, 5 mM potassium chloride and 50 μM
MCD, see also van Schijndel et al., 1993), which compares
very well to the specific activity of approximately 20 U
per of protein that was obtained with purified (see above)
chloroperoxidase from $\underline{C.\ inaequalis}$ itself. The pH activity
15 profiles of the wild type chloroperoxidase and the
recombinant chloroperoxidase derived from yeast are shown
in Figure 3. Figure 3 provides further evidence that the
recombinant yeast produced enzyme has the same
functionality as the wild type enzyme.
20

EXAMPLE 7
Screening for suitable haloperoxidases in other
microorganisms.
25

The microorganisms used in this example are $\underline{Curvularia}$
$\underline{inaequalis}$ (CBS 102.42), $\underline{Drechslera\ biseptata}$ (CBS 371.72),
$\underline{Drechslera\ fugax}$ (CBS 509.77), $\underline{Drechslera\ nicotiae}$ (CBS
655.74), $\underline{Drechslera\ subpapendorfii}$ (656.74), $\underline{Embelisia}$
30 $\underline{hyacinthi}$ (416.71), $\underline{Embelisia\ didymospora}$ (CBS 766,
$\underline{Ulocladium\ chartarum}$ (200.67) and $\underline{Ulocladium\ botrytis}$
(452.72). Various fungi are grown on agar plates. When the
growth is completed the extracellular proteins are
transferred (replica blotted) to a nitrocellulose filter,
35 which was prewetted in 50 mM Tris-HCl buffer (pH 8.3).
After 15 minutes of incubation on the agar plates, the
filter was tested for haloperoxidase activity by soaking
the filter in 100 mM sodium acetate (pH 5.5) or potassium

phosphate (pH 6.5 and 7.5), 1 mM orthodianisidine, 2 mM
hydrogen peroxide in the presence and absence of 0.1 M
potassium bromide. Thus the production of a bromoperoxidase
and/or chloroperoxidase can be detected. To test whether
5   the produced haloperoxidase is a vanadium-containing
haloperoxidase, the test described above was repeated in
the presence and absence of 10 and 100 μM sodium vanadate.
In the case of vanadium-containing haloperoxidases an
increase in signal could be observed in situations where
10   vanadate was supplemented. To test whether the
chloroperoxidases identified are indeed similar to the
vanadium haloperoxidase from C. inaequalis, small amounts
of chloroperoxidases were purified (essentially as
described in van Schijndel et al., 1993) from Ulocladium
15   chartarum, Embelisia didymospora and Drechslera
subpapendorfii. The pH optima of these enzymes varied from
pH 4.5 -5.5. The chlorinating activity of these enzymes
increased upon addition of vanadate, which clearly
indicates that these enzymes are indeed vanadium
20   haloperoxidases. To further test the similarity of the
enzymes identified with C. inaequalis vanadium-
chloroperoxidase, one of the haloperoxidases identified was
further characterised. For this the chloroperoxidase
produced by the fungus Drechslera biseptata (CBS 371.72)
25   was chosen. It has properties similar to the
chloroperoxidase from Curvularia inaequalis that is high
thermostability and high affinity for its substrates. The
EPR spectrum of the purified enzyme was also recorded. As
for other vanadium haloperoxidases (de Boer et al., 1988;
30   Wever et al., 1988), the oxidized enzyme is EPR silent;
however upon reduction with sodium dithionite a typical
vanadyl EPR spectrum was observed (data not shown). The
isotropic EPR parameters g_o of 1.969 and A_0 of 9.0 mT are
almost the same as those found for the enzyme from C.
35   inaequalis (Wever et al, 1985). Furthermore, the purified
enzyme has been split into peptides using proteases and
cyanogen bromide. The peptide maps show the same patterns
suggesting that these two enzymes have a large sequence

homology. This is indeed the case, two separated peptides
of the enzymes which were obtained by treatment of the
enzyme with a protease and which were purified, were
sequenced. The sequences show a very large homology and
5   therefore it can be concluded that the two enzymes are very
similar.

Amino acid sequence of a peptide from C. inaegualis :
(Asp)-leu-arg-gln-pro-tyr-asp-pro-thr-ala-pro-ile-glu-asp-g
10   ln-pro-gly-ile-val-arg-thr-

Amino acid sequence of a similar peptide from D. biseptata

Asp-leu-arg-gln-pro-tyr-asp-pro-thr-ala-pro-ile-glu-glu-gln
15   -pro-gly-ile-val-arg-thr-

Suitable vanadium containing haloperoxidases can thus be
identified by using a replica technique in which the
increase in activity after addition of vanadate is tested,
20   and/or by (partially) purifying the enzyme and looking for
increase in activity after addition of vanadate.

EXAMPLE 8
Further screening for suitable chloroperoxidases in other
25   microorganisms, using antibodies.

The strains used in this example are: Curvularia inaegualis
(CBS 102.42), Drechslera biseptata (CBS 371.72); Drechslera
subpapendorfii (CBS 656.74), Embellissia didymospora (CBS
30   766.79) and Ulocladium chartarum (CBS 200.67).
The microorganisms were grown in two phases. First, 50 ml
of sterile germination medium (as described in Van
Schijndel, et al., 1993) was inoculated with the spore mass
of the microorganisms. The culture was shaken for 3 days at
35   23°C after which the culture was transferred to a 3 litre
Erlenmeyer flask containing 1 litre of fermentation medium
(5 g of casein hydrolysate (Gibco BRL), 3 g of yeast
extract and 1 g of fructose per litre of deionized water).

The medium, which was shaken at 23°C, was collected after
14-17 days, filtered and the chloroperoxidases were
purified essentially according to Van Schijndel et al.
(1994). Polyclonal antibodies were raised (using Freunds
5   complete adjuvans in the first injection and Freunds
incomplete adjuvans in the booster injection) against the
purified (according to van Schijndel et al., 1994)
chloroperoxidase from Curvularia inaequalis in a (2-month-
old female) rabbit. The rabbit was bled 6 days after the
10 last booster injection. The sera were heated for 30 min at
56°C to inactivate complement and then centrifuged. The
supernatant was taken. A dilution series was made of each
of the purified chloroperoxidases and also of
bromoperoxidase from Ascophylum nodosum (purified according
15 to Wever et al, 1985), starting with 50 μl of each protein
(of approximately 0.1 mg per ml), and each sample was
diluted sequentially two times. The dilutions were spotted
using a dot-blot apparatus (Bio-Rad) on a nitrocellulose
filter, washed with 2% BSA and incubated sequentially with
20 a 1:800 dilution of rabbit anti-chloroperoxidase antiserum,
biotinylated goat anti-rabbit (dilution 1:3000), alkaline
phosphatase-conjugated streptavidin (dilution 1:2000) and
colour development reagent (5-bromo-4-chloro-3-indolyl
phosphate, 4-NitroBlue Tetrazolium chloride (Boehringer
25 Mannheim). All steps were performed according to standard
protocols. The results obtained are shown in Table VI.

Table VI

haloperoxidase from	cross-reactivity with polyclonal antibodies raised against C. inaequalis chloroperoxidase
C. inaequalis	yes
D. biseptata	yes
D. subpapendorfii	yes
E. didymospora	yes
U. chartarum	yes
bromoperoxidase from A. nodosum	no

Based on the results disclosed in Table VI it can be concluded that immunoassays with antibodies raised against C. inaequalis chloroperoxidase is suitable to identify other suitable vanadium containing haloperoxidases. These haloperoxidases can be either in crude form, partially or totally purified. Purification techniques can be all techniques known in the art, like gelfiltration, ion exchange chromatography, hydrophobic interaction chromatography, precipitation techniques, (ultra)filtration techniques, affinity chromatography, gelelectrophoresis and others.

EXAMPLE 9
Further screening for suitable chloroperoxidases in other microorganisms.

In this example it si described how a radio-active probe derived from the chloroperoxidase gene from Curvularia inaequalis can be used to detect homologous genes in other microorganisms. This was done as follows:

39

Chromosomal DNA from <u>C. inaequalis</u> (CBS 102.42), <u>Embelissia</u>
<u>didymospora</u> (CBS 766.79) and, <u>Drechlera biseptata</u> (CBS
371.72) was purified essentially as described for <u>C.</u>
<u>inaequalis</u> chromosomal DNA (as described in example 6). For
5 Southern analysis of the genomic DNA, the DNA was digested
with several combinations of restriction enzymes and after
agarose gel electrophoresis blotted to a nitrocellulose
membrane (Sambrook et al., 1989). Hybridization was carried
out using a radiolabeled gene specific fragment, which was
10 made by random priming using alpha-^{32}P labelled dATP
(Sambrook et al. 1989). The gene specific fragment used was
amplified (before radio-active labelling) in a polymerase
chain reaction, using first strand cDNA (see Example 6) as
a template and making use of the primers:
15

5'-CACGATGGGGTCCGTTACAC

and 5'-GTACCGCTATCGCTGCGCCTG

20 The hybridisation conditions were as follows:
In the prehybridisation and hybridisation 6 * SSPE, 5 *
Denhardts, 0.5% SDS and 10 mg salmon sperm DNA were used as
a buffer. Prehybridisation was done for 1 hour at 55°C,
then the radioactive probe was boiled for 1 min and then
25 directly added. Subsequently, hybridisation was continued
overnight. The autoradiograph, which was obtained with
<u>Curvularia inaequalis</u> and <u>Drechlera biseptata</u> DNA is
presented as Figure 4. In the figure, lane 1: lambda DNA;
lane 2: non-digested <u>C. inaequalis</u> genomic DNA; lane 3:
30 idem, digested with EcoRI; lane 4: digested with BamHI;
lane 5: EcoRI and BamHI; lane 6 digested with XbaI; lane 7
PstI; lane 8 XbaI and PstI; lanes 9 - 14: idem, using <u>D.</u>
<u>biseptata</u>. As can be seen in the figure, a positive signal
is obtained with chromosomal DNA from <u>Drechslera biseptata</u>,
35 indicating a high degree of similarity of both genes.
Similar results were obtained with DNA from <u>Embellisia</u>
<u>didymospora</u>. We therefor conclude that the chloroperoxidase
gene, or parts derived from this gene, or probes based on

the sequence of the <u>C. inaequalis</u> chloroperoxidase gene can
be used to detect suitable vanadium haloperoxidases from
other microorganisms.

5   Table VII. Oligonucleotide primers (20mers), based on amino
acid sequences from the vanadium chloroperoxidase ex
<u>Curvularia inaequalis</u>.
I: inosine
A/G: at this position at equal mix of A and G is used.
10 C/T: at this position at equal mix of C and T is used.
G/A/T/C: at this position at equal mix of G, A, C and T is
used.

<u>Oligo 1:</u>
15 5'-T A C/T A T G A A A/G C C I G T I G A A/G C A -3'

<u>Oligo 2:</u>
5'-A G/A T/C T G I G C G/A T A I G C G/A T T G/A T C-3'

20 <u>Oligo 3:</u>
5'-G A C/T G A A/G A C I G C I G A A/G T A C/T G A-3'

<u>Oligo 4:</u>
5'-A G/A I G C T/C T G I G C I C C G/A/T/C C C C A T-3'

25

CLAIMS

1. Enzymatic antimicrobial composition comprising a
5 Vanadium haloperoxidase, a source of halide and hydrogen
peroxide or a source of hydrogen peroxide.

2. Enzymatic antimicrobial composition according to claim
1, wherein the Vanadium haloperoxidase is a
10 chloroperoxidase.

3. Enzymatic antimicrobial composition according to any
one of the preceding claims, wherein the Vanadium
haloperoxidase is a chloroperoxidase obtainable from
15 Curvularia inaequalis

4. Enzymatic antimicrobial composition according to any
one of the preceding claims, wherein the Vanadium
haloperoxidase is a chloroperoxidase which is
20 immunologically cross-reactive with the chloroperoxidase
from Curvularia inaequalis CBS 102.42.

5. Enzymatic antimicrobial composition according to any
one of the preceding claims, wherein the source of hydrogen
25 peroxide is an enzymatic hydrogen peroxide-generating
system.

6. Enzymatic antimicrobial composition according to claim
5, wherein the enzymatic hydrogen peroxide-generating
30 system is glucose/glucose oxidase or lactate/lactate
oxidase.

7. Enzymatic antimicrobial composition according to any
one of the preceding claims, said composition being
35 essentially free from catalase activity.

42

8.   Process for inhibiting microbial growth, comprising
applying a composition according to any one of the
preceding claims to a surface which is to be disinfected.

5   9.   Use of Vanadium haloperoxidases as sanitizing agent.

10. DNA sequence comprising a structural gene coding for
Vanadium chloroperoxidase from <u>Curvularia inaequalis</u> CBS
102.42.

10

11. DNA sequence comprising a structural gene for Vanadium
chloroperoxidase from <u>Curvularia inaequalis</u> CBS 102.42 as
shown in Figure 2.

15 12. Expression vector comprising an origin of replication,
transcription and termination control sequences and at
least part of the DNA sequence according to claim 10 or 11.

13. Process for preparing a Vanadium haloperoxidase by
20 transforming a suitable host by means of an expression
vector according to claim 12, cultivating the host under
conditions which allow the expression of the structural
gene and isolating the Vanadium haloperoxidase.

*****

Fig.1.

Peptide sequences derived from vanadium chloroperoxidases

sequence   cleavage method

C.inaequalis

1ML--LYMKPVEQPNPNPGANISDNAYAQLGLVLDRSVLEA[a]   CNBr

2(S)NADETAEYDDAVRVAIAMGGAQALNS[a]   Trypsin

3(G)YHPTPGRYKFDDEP   Trypsin

4IDEPEEYN   Trypsin

5**(D)LRQPYDPTAPIE**DQPGIVRT[b]   Trypsin

D. biseptata

6LNGLNR**DLRQPYDPTAPIE**EQPGIV[b]   V8 prot.

[a]underlined sequences are used to design degenerated DNA primers.
[b]homologous sequences between *C.inaequalis* and *D. biseptata* are in bold.

Fig.2.

```
ATGGGGTCCG TTACACCCAT CCCACTCCCT AAGATCGATG AACCCGAAGA GTACACACC    60

AACTACATAC TATTCTGGAA CCATGTCGGT TTGGAACTCA ACCGCGTAAC TCACACTGTT   120

GGAGGCCCCC TGACGGGACC ACCTCTCTCT GCCAGGGCTC TGGGTATGCT GCACTTGGCT   180

ATTCACGACG CCTACTTTTC TATCTGCCCT CCGACCGACT TCACCACCTT CCTCTCACCT   240

GATACTGAGA ATGCCCGCTA CCGTCTACCT AGCCCTAATG GTGCAAATGA TGCTCGCCAA   300

GCAGTCGCTG GAGCTGCCCT CAAGATGCTG TCTTCACTGT ACATGAAGCC CGTCGAGCAG   360

CCTAACCCTA ACCCCCGGGC CAACATCTCC GACAACGCTT ATGCTCAGCT TGGCTTGGTT   420

CTCGACCGAT CAGTTCTGGA GGCACCTGGT GGCGTGGACC GAGAGTCAGC CAGTTTCATG   480

TTTGGTGAGG ATGTAGCCGA TGTCTTCTTC GCACTCCTCA ACGATCCTCG AGGTGCTTCG   540

CAGGAGGGCT ACCACCCTAC ACCCGGCCGC TATAAATTTG ACGATGAACC TACTCACCCT   600

GTCGTCCTCA TTCCAGTAGA CCCCAACAAC CCTAATGGTC CCAAGATGCC TTTCCGTCAG   660

TACCACGCCC CATTCTACGG CAAGACCACG AAGCGTTTTG CTACGCCAGAG CGAGCACTTC   720

CTGGCCGACC CACCGGGGCCT GCGTTCTAAT GCGGACGAGA CCGCGGGAGTA TGACGACGCC   780

GTCCGCGTCG CTATCGCCAT GGGTGGTGCT CAGGCTCTCA ACTCCACCAA GCCTAGCCCA   840

TGGCAGACAG CACAGGGCCT ATACTGGGCC TACGATGGGT CAAACCTCAT TGGCACACCA   900
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CCTCGCTTTT ACAACCAGAT CGTACGTCGC ATCGGCAGTTA CGTACAAGAA GGAAGAGGAC   960
CTTGCCAACA GCGAAGTCAA CAATGCGGAT TTCGCCCGCC TCTTCGCCCT CGTCGACGTC  1020
GCTTGCACAG ACGCTGGTAT CTTTTCCTGG AAGGAGAAAT GGGAGTTCGA ATTCTGGCGC  1080
CCACTATCTG GTGTGCCGAGA CGACGGCCGT CCAGACCATG GAGATCCTTT CTGGCTCACT  1140
CTCGGTGCCC CAGCTACTAA CACCAACGAC ATTCCATTCA AGCCTCCTTT CCCAGCTTAC  1200
CCATCTGGTC ACGCGACCTT TGGCGGTGCT GTGTTCCAAA TGGTGCGTCG ATACTACAAC  1260
GGCCGCGTAG GTACATGGAA GGACGACGAA CCCGACACAA TTGCCATCGA TATGATGATC  1320
TCGGAGGAGC TCAACGGCGT GAACCGCGAC CTACGCCAGC CTTATGACCC CACGGCCCCA  1380
ATCGAAGACC AACCCGGTAT CGTGCGCACC CGCATTGTTC GCCACTTCGA CTCGGCCTGG  1440
GAACTCATGT TCGAAAACGC CATTTCGCGC ATCTTCCTCG GTGTCCACTG GCGTTTCGAT  1500
GCCCGCGCCG CCCGCGACAT TCTCATTCCC ACGACGACAA AGGACGTCTA CGCTGTCGAC  1560
AACAATGGCG CCACCGTGTT CCAGAACGTA GAGGACACATTA GGTACACAAC CAGGGGTACG  1620
CGTGAGGACC CCGAGGGCCT CTTCCCTATC GGTGGTGTGC CACTGGGTAT CGAGATTGCG  1680
GATGAGATTT TTAATAATGG ACTTAAGCCT ACGCCCCCGG AGATCCAGCC TATGCCGCAG  1740
GAGACACCGG TGCAGAAGCC GGTGGGACAG CAGCCGGTTA AGGGCATGTG GGAGGAAGAG  1800
CAGGCGCCGG TAGTCAAGGA GGGCGCCGTAG                                  1830
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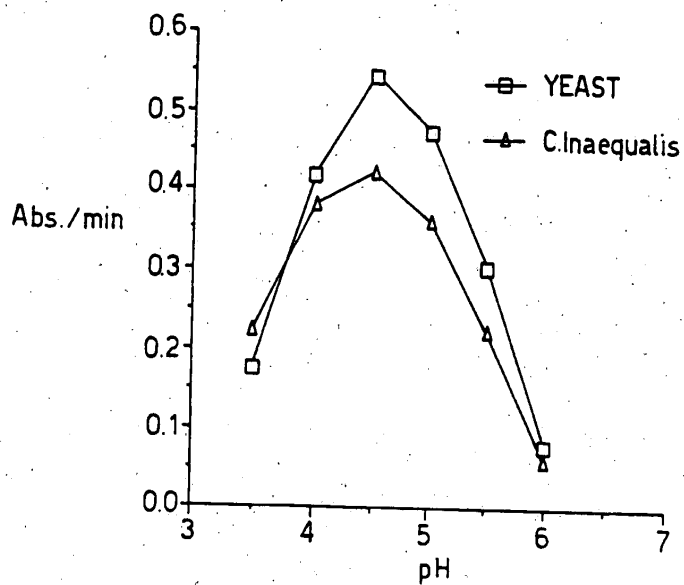
Fig.2. (Cont.)

Fig.3.

Fig.4.